

Confirmation of *Clematis* hybrids using molecular markers

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ABSTRACT

The hybrid origin of progeny from crosses of *Clematis tubulosa* and *Clematis brevicaudata* was investigated using randomly amplified polymorphic DNA (RAPD) and single nucleotide polymorphisms (SNPs) from sequence analysis of chloroplast *rbcl*, *accD* genes, and the *C. brevicaudata* *matK* gene. Plants collected from three and four populations of *C. brevicaudata* (*C. brev*) and *C. tubulosa* (*C. tubu*), respectively, from Mt. Songshan, Beijing, China were used as parents for hybridization. Morphological characters of pollen, seeds, and leaves were recorded in 2007. DNA from leaf samples of both parents and of *C. brev* × *C. tubu* and *C. tubu* × *C. brev* were extracted, and used for RAPD and SNPs from sequence data. A dendrogram was constructed by the branching neighbor-joining (IB-NJ) method. Proportionate population scores were generated by the admixture model using the STRUCTURE software. Based on morphological characters, *C. brevicaudata* was quite uniform. However, variations were detected in *C. tubulosa*. Hybrids of *C. brev* × *C. tubu* and *C. tubu* × *C. brev* showed intermediate morphological characters of the parents. Accessions of *C. tubu* × *C. brev* were clustered into 2 groups, with the majority of hybrids belonging to group IV b in the RAPD dendrogram, suggesting that this resulted from variations within *C. tubulosa*. In general, the hybrid origin of all progeny characterized by morphological characters was supported by the RAPD and SNPs data. These results indicate that RAPD results supported by SNPs data will be useful tool to verify hybrids.

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1. Introduction

The genus *Clematis* includes approximately 210 (USDA, ARS, National Genetic Resources Program, 2008) or 300 (Grey-Wilson, 2000) species. *Clematis* is one of the most popular climbing plants used in landscapes and floriculture as a garden or potted plant (Roh and Song, 1997). Typically, most commercially available cultivars or species will cease flowering during hot weather. Commercial clematis should include genes from previously unused wild species that have tolerance to high temperatures to meet increased demand for new cultivars. More than 90 species of clematis are endemic to high altitudes in Yunnan, Tibet, Gansu, and Sichuan provinces of China, where summer temperatures are low (http://www.efloras.org/florataxon.aspx?flora_id=2&taxon_id=107312, accessed on September 30, 2009).

Clematis brevicaudata DC, in subgenus *Clematis* is a deciduous, heat tolerant woody climber native to wide areas in northern and northeastern China, Korea, and Russia and the Far East and grows at elevations of 460–2800 m. It produces flushes of yellow flowers from July to September and is excellent in gardens as well as covering a fence with blooms. It is common in the countryside around

Beijing, China. This species has not yet been explored and utilized for horticultural use, yet it is a vigorous growing species during hot summer days when compared to other native clematis in China (Grey-Wilson, 2000). *Clematis tubulosa* Turcz. (syn. *C. heracleifolia*) has been popular in gardens since it was introduced into cultivation, produces a hyacinth-like fragrance, and is exceptionally vigorous in hot summers (July to September) in sunny and semi-sunny urban environments in Beijing. *Clematis tubulosa*, subgenus *Tubulosa*, is a non-climbing clematis widely found in China and Korea at elevations of 300–2000 m. Stems are usually branched and grow upright. Flowers are blue to purple in color and fragrant.

Leaf morphology, the growth pattern of seedlings, and pollen characteristics can be used to differentiate hybrid genotypes from parents. The F₁ hybrids of *Camellia sinensis* L. × *Camellia irrawadiensis* Barua showed intermediate morphological characters of the parents (Bezbaruah, 1975). Differences in pollen morphology were noted between *Amaranthus rudis* Sauer and *Amaranthus palmeri* S. Wats. and their hybrids (Franssen et al., 2001). Interspecific hybrids of *Nicotiana sylvestris* Speg. Et Comes × *Nicotiana tabacum* L. showed intermediate vegetative and reproductive morphological characters (Al-Ahmad et al., 2006). Reciprocal hybridizations were made between *C. brevicaudata* × *C. tubulosa* (*C. brev* × *C. tubu*) and *C. tubulosa* × *C. brevicaudata* (*C. tubu* × *C. brev*) to breed upright growing plants with diverse flower shapes and colors. It was not easy to visually verify whether seedlings were truly hybrids and assess-

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ment on heat tolerance could not be determined in relation to the ability of flowering. Additionally, hybrid seedlings require more than 2–3 years to flower so significant time is required to use floral morphology to distinguish hybrid genotypes.

Molecular markers generated from randomly amplified polymorphic DNA (RAPD) (Williams et al., 1990) have been used extensively to study genetic diversity and to confirm hybrids and their parental species, such as in *Juglans cinerea* L., *Berberis thunbergii*, *Mangifera indica* L., *Ardisia crenata* Sims. and *Ilex × wandoensis* (Amy et al., 2008; Anuj et al., 2007; Jessica et al., 2008; Lee et al., 2006; Roh et al., 2006). Single nucleotide polymorphisms (SNPs) (Brookes, 1999) following sequence analysis of various genes, such as the chloroplast gene for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (*rbcL* gene), the chloroplast gene for maturaseK gene (*matK* gene) and other genes in *Clematis* (Miikeda et al., 2006), *Pinus* (Joung and Roh, 2005) and *Corylopsis* (Roh et al., 2007a) have been useful for identification in studies of phylogenetic relationships and genetic variation.

The objectives of this study were to: (a) study the variability of *C. brevicaudata* and *C. tubulosa* and their hybrids by morphological characters of leaves, flowers, seeds, and pollen and (b) identify the putative hybrid origin of young seedlings of progeny resulting from reciprocal crosses of *C. brevicaudata* and *C. tubulosa* using RAPD and SNPs analysis from sequence data of *rbcL* and *matK* genes.

2. Materials and methods

2.1. Plant materials

Plants of *C. brevicaudata* and *C. tubulosa* were randomly selected on Mt. Songshan, Beijing, China, and used for *in situ* hybridization. A total of 10 plants from 3 separate clumps of *C. brevicaudata* in a radius of 1 km and 4 clumps of *C. tubulosa* in a radius of 500 m were collected as indicated in Table 1. Reciprocal hybridization *C. brev* × *C. tubu* and *C. tubu* × *C. brev* were made in August 2005. Pollen collected from all 10 plants of each species was combined for hand pollination. Pollinated flowers were enclosed in paper bags to prevent pollen contamination.

Seeds of *C. tubu* × *C. brev* were collected in October from a single mother plant that produced numerous seeds with high germination rates. Seeds of *C. brev* × *C. tubu* crosses were collected from 3 separate mother plants. Seeds from parents from open pollination and hybrids from controlled pollinations were collected and sown in February 2006. Seedlings were transplanted singly into 8 cm pots filled with peat moss and perlite (3:1 by volume) in a greenhouse, and seedlings were transplanted at Baifu Nursery, Beijing, in September 2006.

2.2. Morphology of leaves, seeds, and pollens

A few parental and hybrid seedlings flowered in August 2007, with morphological characters of leaf was outlined (Fig. 1). A fully developed three-leaflet leaf from the second or third pair of leaves below the inflorescence was collected from *C. tubulosa*. Leaves with 5–7 leaflets from *C. brevicaudata* were collected for measurement. Fully developed five-leaflet leaves from *C. brev* × *C. tubu* and three-leaflet leaves from *C. tubu* × *C. brev* hybrids from the second or third pair of leaves below the inflorescence were sampled for measurement. Data collected from 3 to 6 leaves were subjected to the analysis of variance. Data on flower color using the RHS color chart (Royal Horticultural Society, 1995) were recorded at anthesis.

Fresh pollen was collected from 2 to 3 plants at anthesis for scanning electron microscopy. The collected anthers were air-dried at room temperature for 48 h, then sputter coated with gold–palladium, scanned on the Hitachi S-570 scanning electron

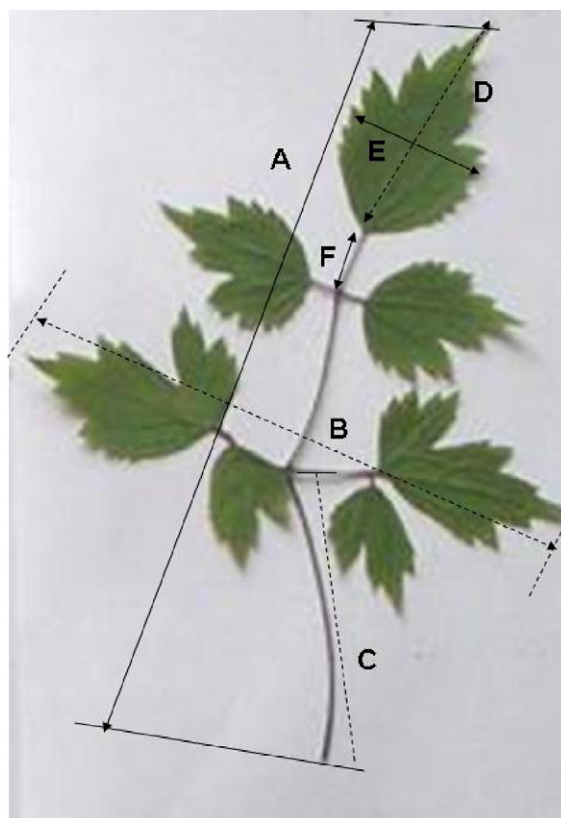


Fig. 1. Measurement of length (A) and width (B) of the entire leaf and of length (D) and width (E) of the terminal leaflet and petiole subtending terminal leaflet (F) and entire leaf (C). This figure does not include any data from Section 3.1.2. It is not stated that these measurements are in cm. If this figure is included in the manuscript these measurements must relate to the data collected.

microscope at an accelerating voltage of 12 kV, and photographed at an amplification magnitude of 3500×. Data on pollen size and morphological traits were collected from 5 pollen grains.

2.3. DNA extraction

Leaf samples for DNA extraction were collected from established seedlings at the nursery; 11 of *C. brev*, 10 of *C. tubu*, 17 of *C. brev* × *C. tubu*, and 38 of *C. tubu* × *C. brev* (Table 1). Total genomic DNA was isolated from dried leaves (50 mg, dry weight) using a DNeasy Plant Mini Kit by QIAGEN (QIAGEN, Inc., Valencia, CA, USA) and quantified by NanoDrop D-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. RAPD analysis

A total of 60 random primers for RAPD (Operon Technologies, Alameda, CA) were screened using 8 selected samples from *C. brevicaudata*, *C. tubulosa*, and hybrids of *C. brev* × *C. tubu* and *C. tubu* × *C. brev*. Thirteen primers (OPA7, OPA9, OPA10, OPA11, OPB4, OPB6, OPB8, OPB10, OPB16, OPC1, OPC4, OPC6, and OPC7) produced polymorphic bands and these were selected for final analyses of all accessions. Polymerase chain reaction (PCR) for RAPD was performed with 1 µl of template (10 ng/1 µl) DNA, 1 µl of 5 pM of primers, 23 µl of PCR grade water using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Pharmacia Biotech Inc., USA) and thermal cycler settings as previously described (Roh et al., 2007b). The molecular size of each band was estimated based on a 100 bp DNA ladder (Fermentas, Hanover, MD).

Table 1
Accessions and morphological characters of *C. brevicaudata*, *C. tubulosa*, and progenies of their reciprocal crosses. Seventy-five samples were used for RAPDs and 45 samples for gene sequencing.

Taxa and hybrids	Accession no. for RAPD	Accession no. for gene sequencing	Remarks
<i>C. brevicaudata</i>	1–10	1, 2, 4, 6, 8, 10	Vine, white-yellowish flower. Collected on Songshan Mt, Beijing. Edge of forest, 850 m for accession 1 and 2; Side of main road, 340 m for accession 3, 4, and 5; and inside forest 870 m for accession 6, 7, 8, 9, and 10.
<i>C. tubulosa</i>	11–20	12, 13, 14, 17, 19,	Non-vining, upright growth. Collected from Songshan Mt, Beijing. Inside forest 870 m for accession 11 and 12, with 4 petaloid calyxes; Roadside along a farmland 840 m for accession 13, 14, and 15, Purple blue flowers, 4 petaloid calyxes, fragrant; underneath of a Chinese walnut tree 840 m for accession 16, 17, and 18, blue flower, 4–7 petaloid calyxes, fragrant; north side of a farmhouse 840 m for accession 19 and 20, dark blue flowers, dwarf form.
<i>C. brevicaudata</i> × <i>C. tubulosa</i>	21–37	21, 23, 25, 26, 28, 30, 34, 37	Baifu Nursery, Beijing, China. Generally showed intermediate characters of the 2 parents.
<i>C. tubulosa</i> × <i>C. brevicaudata</i>	38–75	41–47, 49–51, 53–57, 60, 61, 63, 64, 66, 68–72, 74	Baifu Nursery, Beijing, China. Generally showed intermediate characters of the 2 parents.

2.5. Data analysis

Polymorphic RAPD bands ranging from 400 to 1500 bp were scored as absent (A) or present (T) and the corresponding matrix was used to construct a dendrogram by Molecular Evolutionary Genetics Analysis (MEGA, version 4.0; Tamura et al., 2007). The robustness of the dendrogram was tested by applying bootstrap (BS) with 1000 replications using the interior-branch (IB) neighbor-joining test. To generate proportionate population membership scores for each individual, the admixture model in the STRUCTURE program (Pritchard et al., 2000) was used with the number of clusters (populations) value at 4 (Evanno et al., 2005).

2.6. Sequence analysis and single nucleotide polymorphisms (SNPs)

Based on their sequence information registered at NCBI, primers were designed as described previously by Roh et al. (2008). After screening, three primer sets were selected for SNPs. Primer set 1 (AB116969; *C. brevicaudata* chloroplast *rbcl*, *accD* genes for large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; forward, 5' to 3' TGAGCCGACCCGAATAAATA and reverse, 5' to 3' TTGTGTGAATCGGGGTATCA, product size 529 bp, and annealing temperature at 56 °C), primer set 2 (AB116974; *C. lasiandra* chloroplast *rbcl*, *accD* genes for large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; forward, 5' to 3' CCTATGGATCTTGGGATTGG and reverse, 5' to 3' CGCCCTCC-TATTTGAATGAA, product size 497 bp, and annealing temperature at 57 °C), and primer set 3 (AB110499; *C. brevicaudata* chloroplast gene for maturaseK (*matK*); forward, 5' to 3' TTTGGTCTCAAC-CGGATAGG and reverse, 5' to 3' TTCGGGGGAGAATAAAAGAAA, product size 488 bp, and annealing temperature at 55 °C) were selected and the desirable band for each primer set was sequenced after amplification with Bigdye Terminators (Version 3.1, Applied Biosystems, Foster City, CA). The PCR reaction was performed at

94 °C for 2 min followed by 35 cycles of 94 °C for 15 s, 50 °C for 15 s, and 60 °C for 4 min, and finally at 72 °C for 2 min. The consensus sequence was analyzed by BLAST using the NCBI nucleotide blast program (<http://www.ncbi.nlm.nih.gov/BLAST>).

3. Results

3.1. Characterization of vegetative and reproductive morphological characters

3.1.1. Floral morphology

Clematis brevicaudata plants produced numerous scentless, white-yellowish flowers (RHS color code 8D) and formed vines that grow prostrate to the ground (Figs. 2 and 3). However, *C. tubulosa* plants have an erect habit and produce bluish purple flowers of various colors ranging from bluish purple (RHS color code 88B, 94B, and 96C) with corolla varying in length from 1.8 to 3.5 cm, and width from 0.3 to 1.6 cm (Figs. 2 and 3). Selected hybrids of *C. brev* × *C. tubu* and *C. tubu* × *C. brev* showed intermediate phenotype both in flower color and morphology (Fig. 4). Flower color of *C. brev* × *C. tubu* hybrids varied from RHS 12D (frames 1 and 2) to N80D and N81D (frame 5), and 88D (frame 4). The presence of bluish purple (frame 3, RHS N88C) to white-yellowish flower color resulted from *C. tubulosa*. All of *C. brev* × *C. tubu* hybrids had slight fragrance and showed creeping or climbing growth characteristics. Selected hybrids of *C. tubu* × *C. brev* also showed intermediate phenotype both in flower color and shape. Also, all hybrids showed creeping or climbing growth characteristics and the flower color varied from RHS N80D (frame 7) and N80C (frame 8) to N84B (frame 12), and N88D (frame 9).

3.1.2. Leaflets and leaf morphology

The number of leaflets per leaf ranged from 3.0 for *C. tubulosa* with typically ternate leaves to 7.5 for *C. brevicaudata* with typical twice-ternate leaves. The number of leaflets of *C. brev* × *C. tubu* and *C. tubu* × *C. brev* hybrids was 4.3 and 6.5, respectively

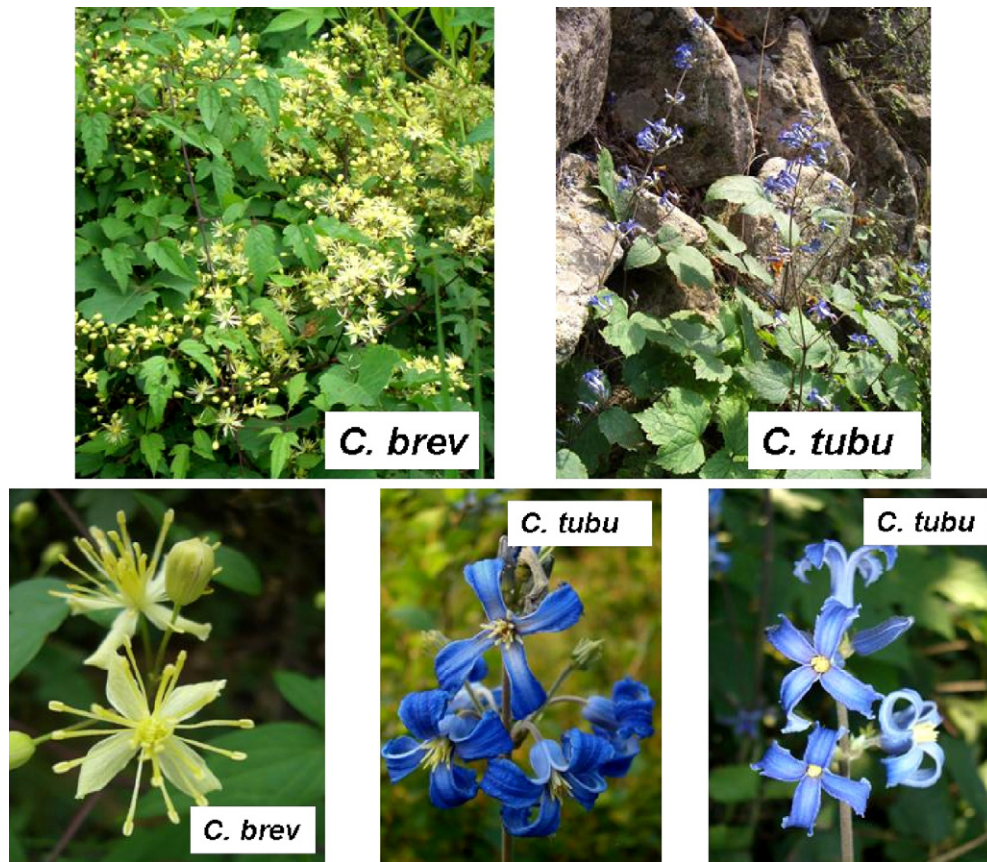


Fig. 2. Plants at flowering and close-ups of the inflorescence of *C. brevicaudata* (*C. brev*) and *C. tubulosa* (*C. tubu*). Two types of flowers of *C. tubulosa* differing in color and shape of petals are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

(Table 2). It was difficult to count the number of leaflets in *C. brevicaudata* because some leaflets were separated by a very short petiole (<0.3 cm) at the base of each leaflet. Generally, the length (21.5 cm) and width (11.3 cm) of the entire leaf of *C. tubulosa* was

significantly greater than that of *C. brevicaudata*, and similar trends were also observed in the terminal leaflet and petiole length. The general shape of each leaflet and the entire leaf (Fig. 5) exhibited the intermediate morphological characteristics of *C. tubu* × *C. brev*.



Fig. 3. Close-ups of *C. brevicaudata* (frame 1, RHS 8D) flower which is uniform in color and shape, and of *C. tubulosa* flowers which vary in shape and flower color (frame 2, RHS 68A; frame 3, 88B; frame 4, 94B; frame 5, 96C; frame 6, 88B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

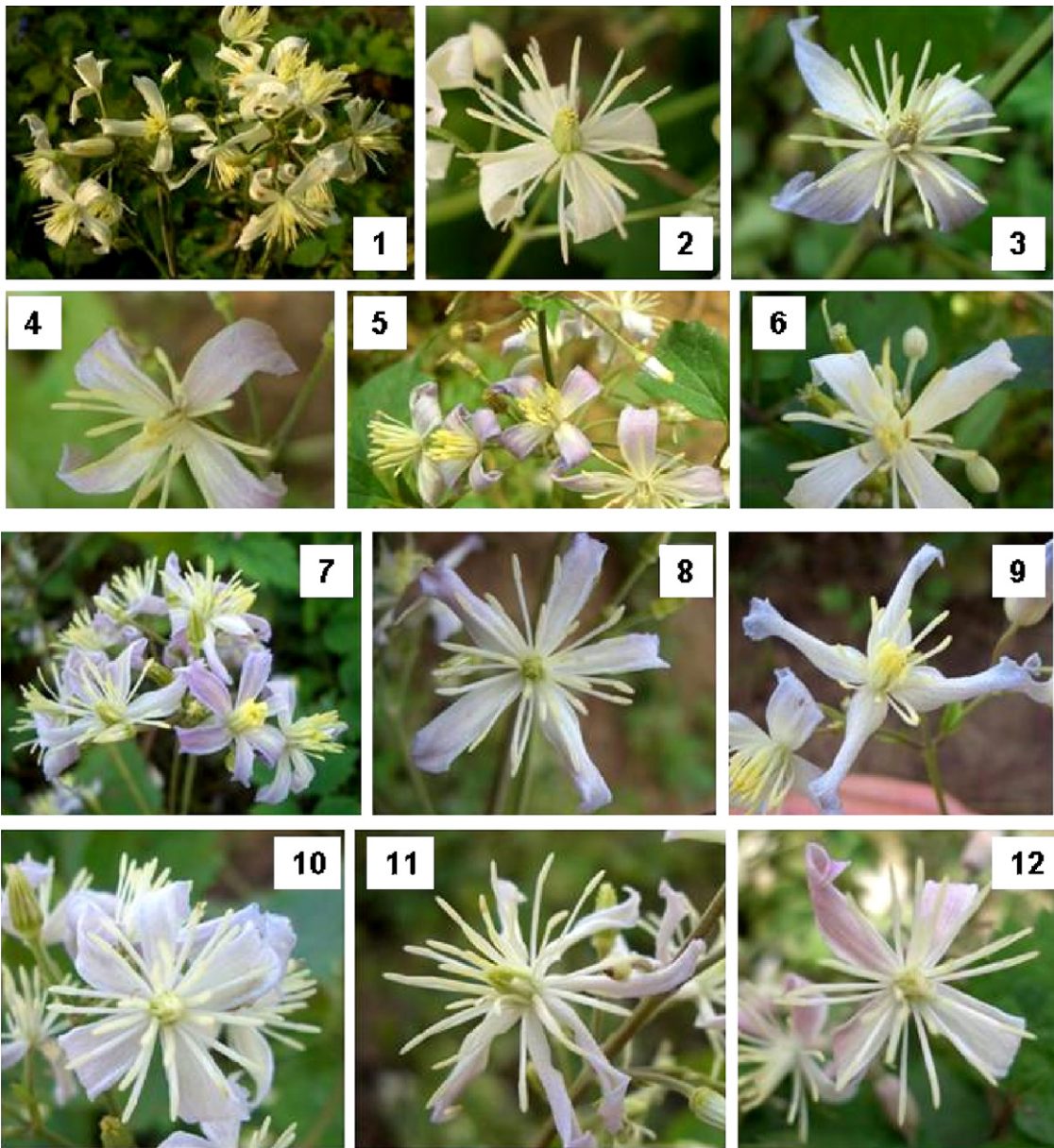


Fig. 4. Inflorescence and various shapes and colors of flowers of *C. brev* × *C. tubu* (frames 1 through 6) and of *C. tubu* × *C. brev* (frames 7 though 12). Photos of frames 1 and 7 show a typical inflorescence shape.

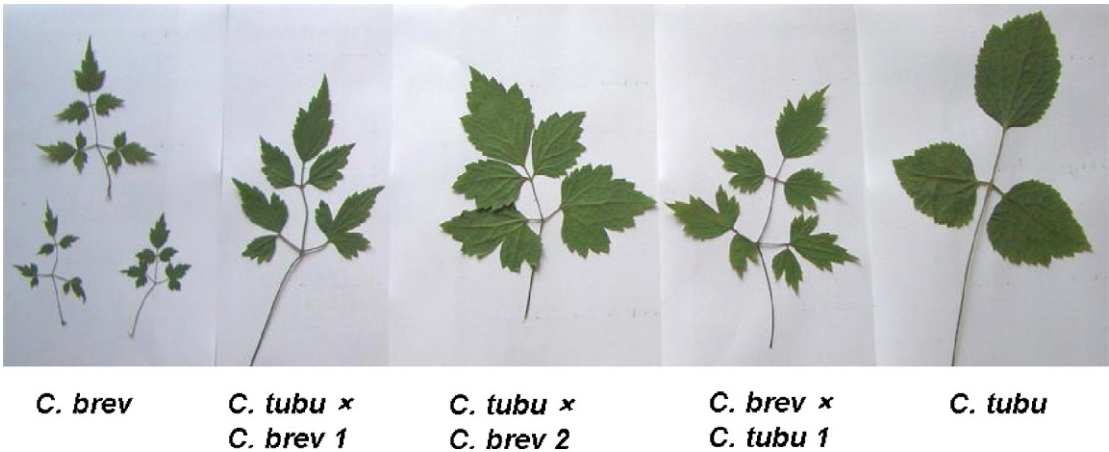


Fig. 5. Leaf morphology of *C. brevicaudata* (*C. brev*), *C. tubulosa* (*C. tubu*) and hybrids of *C. brev* × *C. tubu* and *C. tubu* × *C. brev*. Typical variation of leaflet morphology of *C. tubu* × *C. brev* is shown.

The broadly ovate leaf base in *C. tubu* × *C. brev* 2 and the 4–6 lobate margins on each side were traits that could be traced to *C. tubulosa* (Fig. 5).

3.1.3. Seed and pollen

Seeds of *C. brevicaudata* are smaller than those of *C. tubulosa*, and their hybrids showed an intermediate size. The average length of seeds × width of seeds × length of the persistent feathery styles was 2.5 mm × 1.6 mm × 10 mm for *C. brevicaudata*, 3.6 mm × 2.2 mm × 17 mm for *C. brev* × *C. tubu*, 4.2 mm × 2.6 mm × 21 mm for *C. tubu* × *C. brev*, and 4.7 mm × 2.4 mm × 21 mm for *C. tubulosa*. From the fine structure on the exine small pores around echinae were not observed in *C. brevicaudata*, but they were observed in *C. tubulosa* and also in *C. brev* × *C. tubu* and *C. tubu* × *C. brev* (Fig. 6).

3.2. Confirmation of interspecific hybrids using molecular markers

3.2.1. RAPD analysis – clustering and STRUCTURE

On the average, 7.6 polymorphic bands were produced per primer (data not presented). Based on the dendrogram developed with molecular markers generated by RAPDs (RAPD dendrogram) and constructed by the neighbor-joining method using the interior-branching test (IB-NJ) (Fig. 7), all *C. tubulosa* accessions, except *C. tubu* × *C. brev* hybrid 68 and possibly one parent of hybrid 12, clustered in group I and all *C. brevicaudata* accessions clustered in group II. All *C. brev* × *C. tubu* hybrids except accessions 26 and 32 formed a group III. However, hybrids of *C. tubu* × *C. brev* were clustered in 2 groups, group IV a and IV b. Hybrids of *C. tubu* × *C. brev* 55 and 56 were clustered together with group III of the *C. brev* × *C. tubu* hybrids. Bands inherited from both parental taxa (*C. tubulosa* indicated by band a and *C. brevicaudata* indicated by band b) were evident (Fig. 8). For example, both bands were present in *C. brev* × *C. tubu* accession 24 with * mark and *C. tubu* × *C. brev* (i.e., accession 25 with ** mark). Another marker band of ca. 1300 bp for *C. tubulosa* (not indicated in figure) was evident.

Based on STRUCTURE analysis, it is evident that all *C. brev* × *C. tubu* hybrids showed posterior probability scores greater than 0.95 (95%, numerical data not presented) of *C. brevicaudata* in all accessions (Fig. 9), corresponding to the resolution of accessions in the RAPD dendrogram. *Clematis tubu* × *C. brev* hybrid accession 55 and 56 could belong to group III based on STRUCTURE analysis. Although some accessions (39, 40, 44, and 63 of *C. tubu* × *C. brev* hybrids) clustered with Group IV b in the RAPD dendrogram, 100% posterior probability values that were shown in *C. brev* × *C. tubu* hybrids can be grouped with group III.

3.2.2. Single nucleotide polymorphisms (SNPs)

A BLAST search of the consensus sequences of three genes (*C. brevicaudata* chloroplast *rbcl*, *C. lasiantha* chloroplast *rbcl*, and *C.*

brevicaudata chloroplast gene for *matK*) matched (>99%) with gene sequence information registered at NCBI (data not presented). Only one SNP position was discovered in each of the genes; position 415, 252, and 293 for *C. brevicaudata* *rbcl*, *C. lasiantha* *rbcl*, and *C. brevicaudata* *matK*, respectively (Table 3). All *C. brevicaudata* parents showed the same modification at the specific SNP position giving either T or A, and all *C. tubulosa* parents showed either G or C, depending on the gene sequenced. All *C. tubu* × *C. brev* hybrids showed G, G, and C at position 415, 253, and 293, respectively, for the respective genes (Table 3).

4. Discussion

4.1. Success of hybridization and morphological characters

The natural population of *C. brevicaudata* parents collected at 2 altitudes on Mt. Songsan is considered more uniform based on the morphological characters of leaves and especially flower color and shape than the *C. tubulosa* parents collected at similar altitudes. Corolla color and width variations of *C. tubulosa* were observed. This suggests that there is a certain level of genetic variation, and selecting *C. tubulosa* for a breeding program should be done cautiously. For example, breeding for wide corolla characteristics would require selection of an accession such as *C. tubulosa* as shown in frame 4 in Fig. 3, under the assumption that this trait will be inherited through sexual propagation. The slight fragrance in *C. brev* × *C. tubu* hybrids could be inherited from *C. tubulosa*, and prostrate growth characteristics from *C. brevicaudata*, which confirms the hybrid origin based on morphological characters. Interspecific hybrids of *N. sylvestris* Speg. Et Comes × *N. tabacum* L. showed intermediate vegetative and reproductive morphological characters which are close to those observed in the male parent (Al-Ahmad et al., 2006).

C. tubulosa seeds are largest, *C. brevicaudata* seeds are the smallest, and the hybrids are intermediate between the two parents. This and the fact that leaf size and shape of hybrids also are intermediate between the parents, indicates that progenies of both reciprocal crosses resulted from interspecific hybridization. The success of hybridization was further supported in the fine structure of echinae both in *C. tubulosa* and hybrids of *C. brev* × *C. tubu* and also *C. tubu* × *C. brev*. The presence of small pores both in *C. tubulosa* and hybrids also support the hypothesis of a successful hybridization. Similar intermediate phenotypes were seen in interspecific hybrids in other genera. The morphological and anatomical characters in the F₁ hybrids of *C. sinensis* L. × *C. irrawadiensis* Barua (Bezbaruah, 1975) and of *Ilex* × *wandoensis* C.F. Mill., and M. Kim (Lee et al., 2006) were intermediate between parents. Pollen grain morphology of *C. brev* × *C. tubu* and also *C. tubu* × *C. brev* hybrids, in general, is similar to that of *C. tubulosa*, suggesting that it could be controlled by *C. tubulosa*. Differences in pollen morphology noted between *A. rudis* Sauer and *A. palmeri* S. Wats. and their hybrids indicate that pollen

Table 2

Morphological data of leaves and leaflets of *C. brevicaudata* (*C. brev*), *C. tubulosa* (*C. tubu*) and hybrids of *C. brev* × *C. tubu* and *C. tubu* × *C. brev*.

Species and hybrids	No. of leaflets per leaf	Entire leaf		Terminal leaflet		Petiole length (cm)	
		Length, A ^a (mm)	Width, B (mm)	Length, D (mm)	Width, E (mm)	Leaf, C	Leaflet, F
<i>C. brevicaudata</i>	7.5 ± 0.96 ^b	13.1 ± 0.77	7.3 ± 0.50	4.3 ± 0.82	2.5 ± 0.32	6.6	0.6
<i>C. tubulosa</i>	3.0 ± 0.00	21.5 ± 2.25	11.3 ± 0.48	9.2 ± 0.68	6.1 ± 0.64	8.4	2.6
<i>C. brev</i> × <i>C. tubu</i>	6.5 ± 0.96	17.0 ± 2.43	9.9 ± 1.88	5.6 ± 0.51	3.4 ± 0.45	7.7	1.3
<i>C. tubu</i> × <i>C. brev</i>	4.3 ± 0.42	20.3 ± 1.16	12.0 ± 0.74	5.3 ± 0.60	3.3 ± 0.64	8.6	1.1
Level of significance	***	**	**	**	**	ns	ns

ns, not-significant.

^a Refer to Fig. 1 for details how the data were measured.

^b Mean ± standard error.

** Significant at 1% (F-test).

*** Significant at 0.1% (F-test).

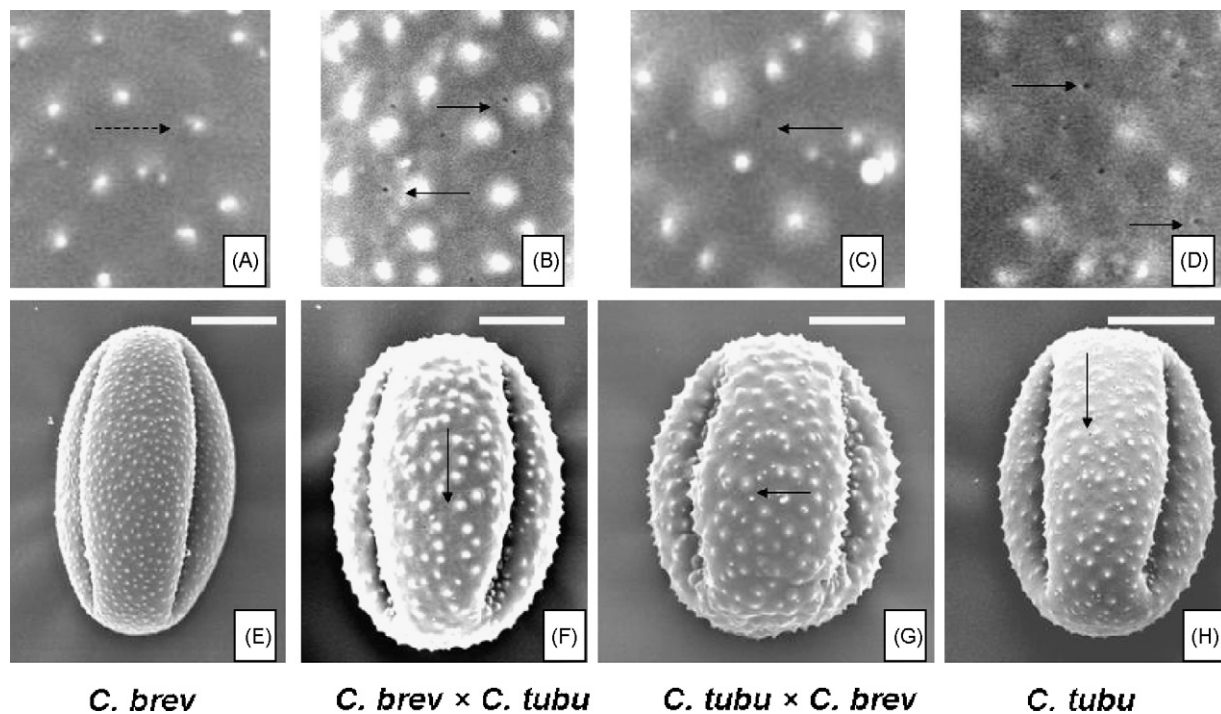


Fig. 6. Scanning electron microscopy of pollen morphology of *C. brevicaudata* (frame E), *C. tubulosa* (frame H), and their hybrids, *C. brev* × *C. tubu* (frame F) and *C. tubu* × *C. brev* (frame G) showing fine structure of echinae (broken arrow, frame A) and small pores (solid arrows, frame B, C, D, F, G, and H). Bar = 8.6 μm. Magnification at 3500×.

characteristics may be controlled by the female parent (Franssen et al., 2001).

There is no report that *C. brevicaudata* was utilized in a clematis breeding to our knowledge. *Clematis tubulosa* is, however, known to hybridize relatively easily with different species in the same sub-genus, such as *C. stans* Sieb. & Zucc. and *C. vitalba* L. (Grey-Wilson, 2000). Some of the hybrids are more like *C. tubulosa*, bearing a scented, dense flower cluster. The increase in the number of flowers per inflorescence in seedlings of *C. tubu* × *C. brev* that flowered (data not presented) is inherited from the multi-floriferous characters of *C. brevicaudata*. Vegetatively propagated seedlings produced from seedlings should be tested to further evaluate for horticultural merits, such as heat tolerance and upright growth characters at flowering. Since both parents thrive and flower well during hot weather, it is expected that all hybrids would perform well.

4.2. Confirmation of genetic variations and hybridization

The success of interspecific hybridization based on morphological characters is supported by molecular markers. Clustering of *C. tubu* × *C. brev* hybrids into 2 sub-groups (IV a and IV b) in RAPD dendrogram may result from the heterogeneous nature of *C. tubulosa*. However, clustering of *C. tubulosa* accession 12 suggests that this accession could be of hybrid origin, based on the posterior probability value analyzed by STRUCTURE. *Clematis tubulosa* accession 12 may result from the cross between *C. brev* × *C. tubu* or expression of genes from *C. brevicaudata* may be dominant to those of *C. tubulosa*. In addition to accession 12, clustering of accession 68 (hybrid of *C. tubu* × *C. brev*) in the RAPD dendrogram suggests that this probably resulted from a back-cross to *C. tubulosa*. In tomato (*Solanum lycopersicon* L.), 10 out of 208 F₁ hybrids proved to be false hybrids using

Table 3

Single nucleotide polymorphisms of *C. brevicaudata* (*C. brev*), *C. tubulosa* (*C. tubu*) and hybrids of *C. brev* × *C. tubu* and *C. tubu* × *C. brev*.

Primer	SNP position	Species or hybrids	Modification	Accession number ^a
Primer 1; <i>C. brev</i> chloroplast <i>rbcl</i>	415	<i>C. brevicaudata</i>	T	1, 2, 4, 6, 8, 10
		<i>C. tubulosa</i>	G	12, 13, 14, 17, 19
		<i>C. brev</i> × <i>C. tubu</i>	T	21, 23, 25, 26, 28, 30, 34, 37
		<i>C. tubu</i> × <i>C. brev</i>	G	41, 42, 43, 44, 45, 46, 47, 49, 50, 51, 53, 54, 55, 56, 57, 60, 61, 63, 64, 66, 68, 69, 70, 71, 72, 74
Primer 2; <i>C. lasiandra</i> chloroplast <i>rbcl</i>	252	<i>C. brevicaudata</i>	T	1, 2, 4, 6, 8, 10
		<i>C. tubulosa</i>	G	12, 13, 14, 17 (19 ^b)
		<i>C. brev</i> × <i>C. tubu</i>	T	21, 23, 25, 26, 28, 30, 34, 37
		<i>C. tubu</i> × <i>C. brev</i>	G	41, 42, 43, 44, 45, 46, 47, 49, 50, 51, 53, 54, 55, 56, 57, 60, 61, 63, 64, 66, 68, 69, 70, 71, 72, 74
Primer 3; <i>C. brev</i> chloroplast gene for <i>matK</i>	293	<i>C. brevicaudata</i>	A	1, 2, 4, 6, 8, 10
		<i>C. tubulosa</i>	C	12, 13, 14, 17, 19
		<i>C. brev</i> × <i>C. tubu</i>	A	21, 23, 25, 26, 28, 30, 34, 37
		<i>C. tubu</i> × <i>C. brev</i>	C	41, 42, 43, 44, 45, 46, 47, 49, 50, 51, 53, 54, 55, 56, 57, 60, 61, 63, 64, 66, 68, 69, 70, 71, 72, 74

^a Refer to Table 1 for accession information.

^b Reaction was not successful for accession 19.

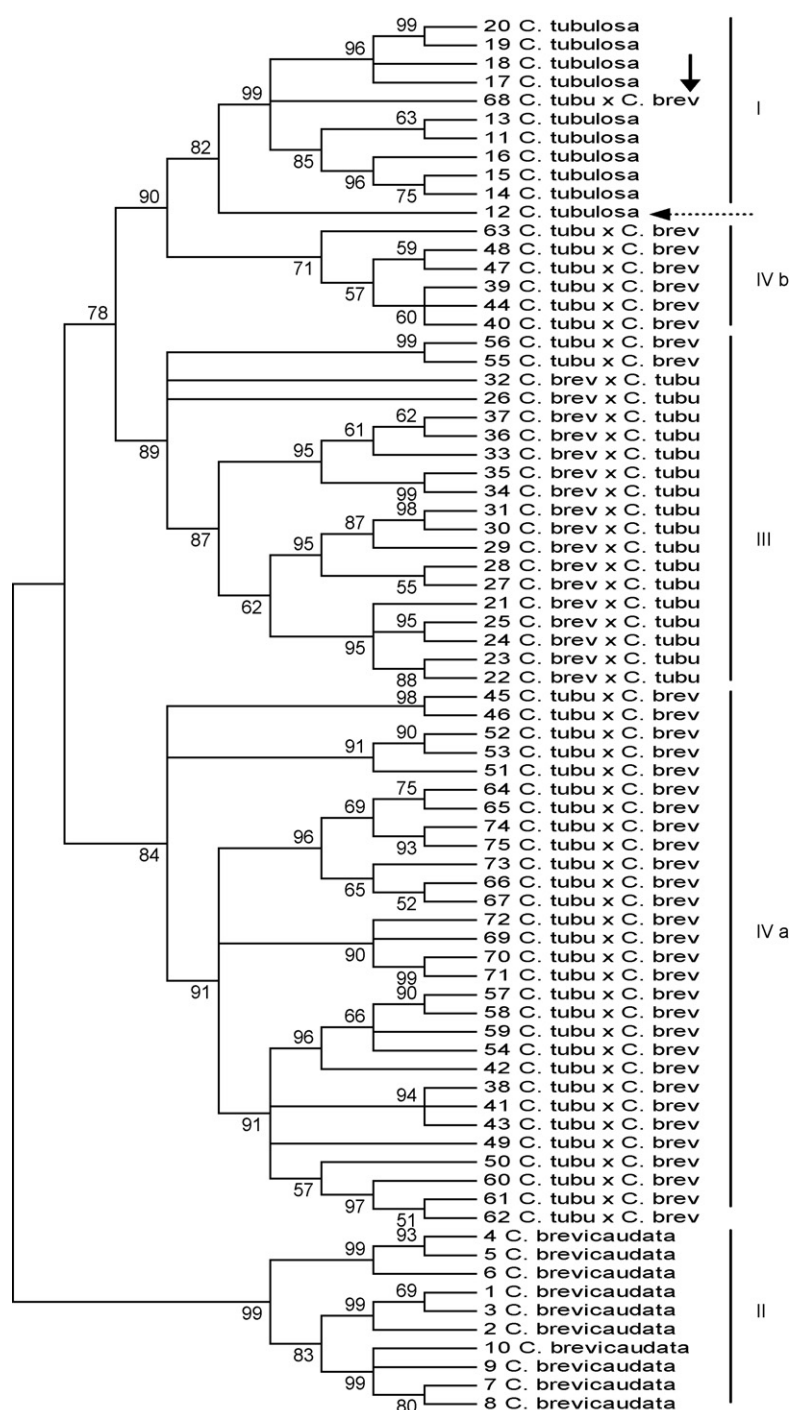


Fig. 7. Dendrogram constructed using molecular markers generated for RAPDs following neighbor-joining method by interior-branching test. A cut-off bootstrap value was set at 60 following 1000 replicates are given at each node.

molecular markers (Liu et al., 2008). The possibility of introgression of hybrids with parental plants requires a determination of whether pollen of *C. brev* × *C. tubu* or *C. tubu* × *C. brev* is viable or not.

Single nucleotide polymorphisms analysis of 3 genes did not support clustering of group IV into 2 or 3 groups, showing the respective modification of nucleotide at the same SNP position and confirming the successful hybridization between *C. brevicaudata* and *C. tubulosa*. Reproducibility is often discussed in an analysis of data from RAPDs (Jones et al., 1997; Penna et al., 1993). RAPDs are, however, a simpler, viable, and more convenient tool than the analysis of SNPs used in this study. RAPDs effectively verify

the hybrid nature of a cross and detect genetic variability of both parental plants. A similar result was obtained in the identification of unknown accessions of *Hydrangea* that were grown sympatrically with *Schizophragma hydrangeoides* Sieb. & Zucc. and *Hydrangea anomala* subsp. *petiolaris* (Sieb. & Zucc.) E.M. McClint (Roh, unpublished data).

In conclusion, *C. brevicaudata* and *C. tubulosa* have been successfully hybridized as determined morphologically and also by using molecular markers generated by RAPDs and SNPs. The reason for clustering of *C. tubu* × *C. brev* into 2 groups in a RAPD dendrogram and into 3 groups in an AFLP dendrogram must be investigated fur-

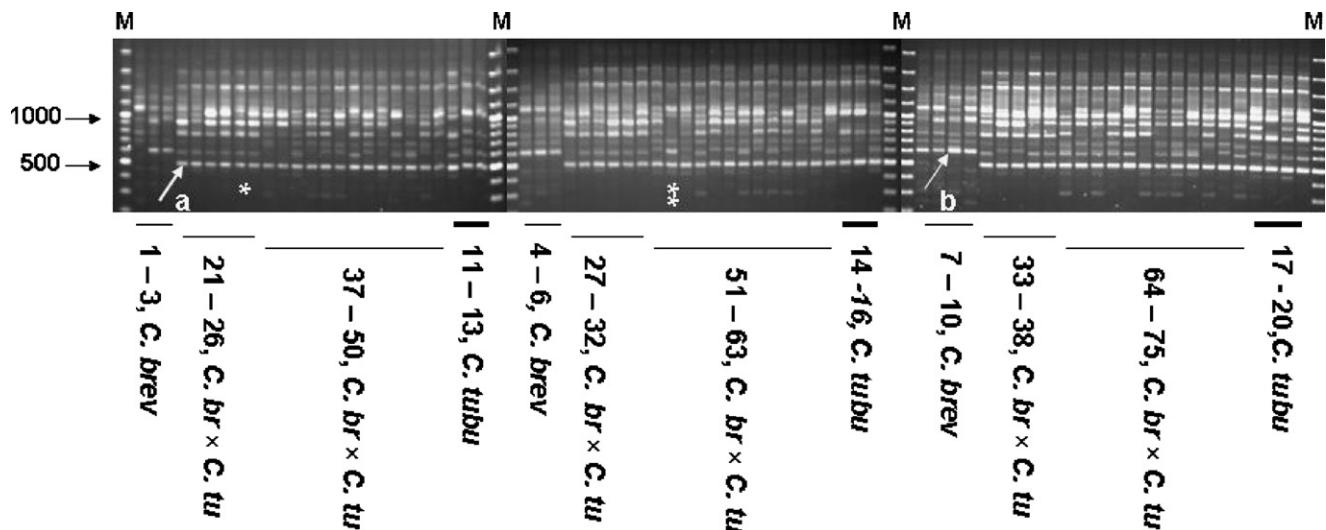


Fig. 8. Gel photograph of amplified fragments from *C. brevicaudata* (*C. brev*, *C. br*), *C. tubulosa* (*C. tubu*, *C. tu*), and hybrids of *C. br* × *C. tu* and *C. tu* × *C. br* using C 07 Operon primers. Accession number of each parent and hybrids are indicated by number. Bands responsible for male parent (arrow indicated by a) and female parent (arrow indicated by b), and selected accession of *C. br* × *C. tu* (*, accession 25) and *C. tu* × *C. br* (**, accession 52) showing both male and female marker bands are indicated. M: DNA ladder, with 500 and 1000 bp. Refer to Table 1 for details of accession for parents and hybrids.

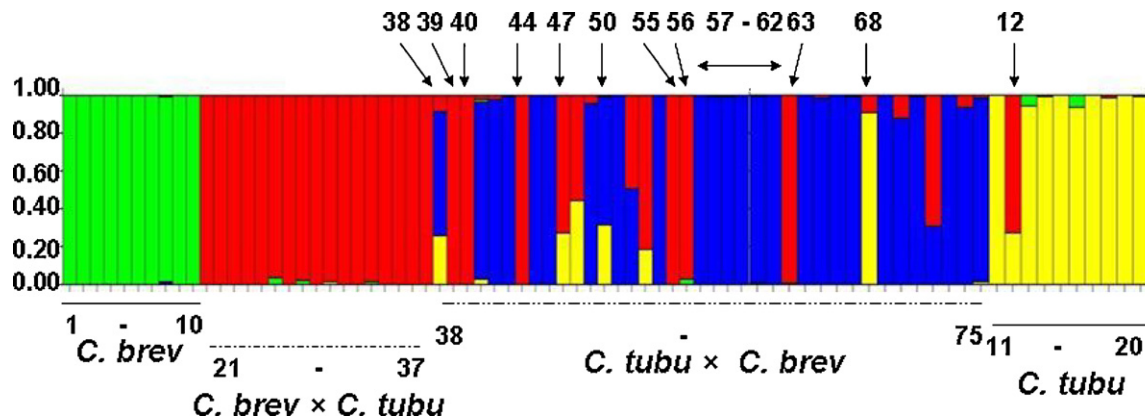


Fig. 9. Posterior probabilities of the 2 parental taxa *C. brevicaudata* (*C. brev*) and *C. tubulosa* (*C. tubu*) and progenies of 2 hybrids resulting from reciprocal crosses using STRUCTURE program based on RAPD markers. Refer to Table 1 for details of accession for parents and hybrids.

ther, but most likely results from the variations observed within *C. tubulosa*.

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